A42
C004

$C_{43}H_{88}N_4O_3.2CF_3CO_2H$ and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl) pyridinium chloride.

REMARKS

Any fees that may be due in connection with this application throughout its pendency may be charged to Deposit Account No. 50-1213.

The specification is amended to correct typographical and spelling errors and to produce grammatical clarity. The specification is also amended to delete the inadvertently typed letters "WP" and to add the inadvertently omitted word "cytometry". The specification is also amended to delete the inadvertently typed letter "d" and to add the inadvertently omitted word "cytometry". The basis for these amendments is found in the specification, in particular page 6, line 23, which describes flow cytometry as a detection method. The specification is also amended to add the inadvertently omitted phrase "of a nucleic acid molecule can occur" for grammatical clarity. The basis for this amendment is found in the specification, in particular page 8, lines 11-12, which describes delivery of a nucleic acid molecule. The specification is also amended to correct the names of chemical compounds. The bases for these amendments are found in the specification, in particular page 7, line 5, for iododeoxyuridine and bromodeoxyuridine and page 20, lines 9-13, for dioleoylphosphatidylethanolamine, dipalmitoylphosphatidylethanolamine, phosphatidylethanolamine, and phosphatidylcholine. The specification is also amended to delete the second occurrence of the word "dipalmitoylphosphatidylethanolamine" on page 20, line 11, to remove redundancy and produce grammatical clarity.

The amendments to claims 6, 8, 39, 42, 53, 59, 67, 71, 140, and 141 correct typographical and spelling errors and produce grammatical clarity. The amendments to claims 14, 36, 51, 79, 87, 94, 102, 110, 118, 138, and 143 delete the second occurrence of the word "dioleoylphosphatidylethanolamine (DOPE)" to remove redundancy and produce grammatical clarity. The amendments to claims 89, 96, 104, 112, 120, 127,

and 133 correct typographical errors and find basis on page 8, line 29, of the specification and in claim 30. No new matter has been added.

Included as an attachment is a marked-up version of the specification paragraphs that are being amended, per 37 CFR §1.121.

* * *

Entry of this amendment and examination of the application are respectfully requested.

Respectfully submitted,
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: De Jong *et al.*

Serial No.: 09/815,979
Confirmation No. 7635
Filed: March 22, 2001

For: *METHODS FOR DELIVERING NUCLEIC ACID MOLECULES INTO CELLS AND ASSESSMENT THEREOF*

Art Unit: Unassigned

Examiner: Unassigned

ATTACHMENT TO THE PRELIMINARY AMENDMENT MARKED UP PARAGRAPHS AND CLAIMS (37 CFR §1.121)

IN THE SPECIFICATION:

Please amend the specification as follows:

Please amend the paragraph on page 1, lines 3-8, as follows:

RELATED APPLICATIONS

This application is [a divisional of] related to U.S. application Serial No. 09/815,979 [(attorney dkt. no. 24601-416)], to de Jong *et al.*, entitled "METHODS FOR DELIVERING NUCLEIC ACID MOLECULES INTO CELLS AND ASSESSMENT THEREOF." The subject matter thereof is incorporated in its entirety by reference thereto.

Please amend the paragraph on page 5, lines 3-7, as follows:

Included among the nucleic acid molecules that may be delivered into cells using the methods provided herein are artificial chromosomes, satellite DNA-based artificial chromosomes (SATACs, herein referred to as [ACEs]ACes) and natural chromosomes or fragments of any of these chromosomes.

Please amend the paragraph on page 7, lines 8-13, as follows:

Because of the ease with which numbers of events are collected, the monitoring methods provided herein, particularly those based on flow [WP]

cytometry techniques, provide a method for collection of nucleic acid molecule delivery data that is statistically superior to previous methods of evaluating nucleic acid molecules transfer. The positive values are instrument derived and therefore are not susceptible to judgement errors.

Please amend the paragraph on page 8, lines 11-30, as follows:

In particular embodiments, the methods of monitoring delivery and expression of a nucleic acid molecule include the steps of: introducing labelled nucleic acid molecules that encode a reporter gene into cells;
detecting labelled cells as an indication of delivery of the nucleic acid into a cell; and measuring the product of the reporter gene as an indication of DNA expression in the cell, whereby delivery and expression of nucleic acid molecules in the cell is detected or determined. The labelled cells can be detected, for example, by flow [d]cytometry, fluorimetry, cell imaging or fluorescence spectroscopy. The label, for example, can be iododeoxyuridine (IdU or IdUrd) or bromodeoxyuridine (BrdU), the reporter gene, for example, can be one that encodes fluorescent protein, enzyme, such as a luciferase, or antibody. The delivered nucleic acid molecules include, but are not limited to, RNA, including ribozymes, DNA, including naked DNA and chromosomes, plasmids, chromosome fragments, typically containing at least one gene or at least 1 Kb, naked DNA, or natural chromosomes. The method is exemplified herein by determining delivery and expression of artificial chromosome expression systems ([Aces]ACes). Any types of cells, eukaryotic and prokaryotic, including cell lines, primary cell lines, plant cells, and animal cells, including stem cells, embryonic cells, and other cells into which delivery of a nucleic acid molecule can occur is contemplated.

Please amend the paragraph beginning on page 13, line 26, through page 14, line 7, as follows:

As used herein, cationic compounds are compounds that have polar groups that are positively charged at or around physiological pH. These compounds facilitate delivery of nucleic acid molecules into cells[,]; it is thought this is achieved by virtue of their ability to neutralize the electrical charge of nucleic acids. Exemplary cationic compounds include, but are not limited to, cationic lipids or cationic polymers or mixtures thereof, with or without neutral lipids, polycationic lipids, non-liposomal forming lipids, ethanolic cationic lipids and cationic amphiphiles. Contemplated cationic compounds also include activated dendrimers, which are spherical cationic polyamidoamine polymers with a defined spherical architecture of charged amino groups which branch from a central core and which can interact with the negatively charged phosphate groups of nucleic acids (e.g., starburst dendrimers).

Please amend the paragraph beginning on page 15, line 18, through page 16, line 4, as follows:

As used herein, gene therapy involves the transfer or insertion of nucleic acid molecules, and, in particular, large nucleic acid molecules, into certain cells, which are also referred to as target cells, to produce specific gene products that are involved in correcting or modulating diseases or disorders. The nucleic acid is introduced into the selected target cells in a manner such that the nucleic acid is expressed and a product encoded thereby is produced. Alternatively, the nucleic acid may in some manner mediate expression of DNA that encodes a therapeutic product. This product may be a therapeutic compound, which is produced in therapeutically effective amounts or at a therapeutically useful time. It may also encode a product, such as a peptide or RNA, that in some manner mediates, directly or indirectly, expression of a therapeutic product. Expression of the nucleic acid by the target cells within an organism afflicted with a disease or disorder thereby [providing] <u>provides</u> a way to modulate the disease or

disorder. The nucleic acid encoding the therapeutic product may be modified prior to introduction into the cells of the afflicted host in order to enhance or otherwise alter the product or expression thereof.

Please amend the paragraph beginning on page 16, line 24, through page 17, line 8, as follows:

As used herein, a reporter gene construct is a DNA molecule that includes a reporter gene operatively linked to a transcriptional control sequence. The transcriptional control sequences include the promoter and other optional regulatory regions, such as enhancer sequences, that modulate the activity of the promoter, or control sequences that modulate the activity or efficiency of the RNA polymerase that recognizes the promoter, or control sequences that are recognized by effector molecules, including those that are specifically induced by interaction of an extracellular signal with a cell surface protein. For example, modulation of the activity of the promoter may be effected by altering the RNA polymerase binding to the promoter region, or, alternatively, by interfering with initiation of transcription or elongation of the mRNA. Such sequences are herein collectively referred to as transcriptional control elements or sequences. In addition, the construct may include sequences of nucleotides that alter translation of the resulting mRNA, thereby altering the amount of reporter gene product.

Please amend the paragraphs beginning on page 19, line 24, through page 20, line 18, as follows:

Cationic Compounds

Cationic compounds for use in the methods provided herein are available commercially or can be synthesized by those of skill in the art. Any cationic compound may be used for delivery of nucleic acid molecules, such as DNA, into a particular cell type using the provided methods. One of skill in the art by using the provided screening procedures can readily determine which of the

cationic compounds are best suited for delivery of specific nucleic acid molecules, such as DNA, into a specific target cell type.

(a) Cationic Lipids

Cationic lipid reagents can be classified into two general categories based on the number of positive charges in the lipid headgroup; either a single positive charge or multiple positive charges, usually up to 5. Cationic lipids are often mixed with neutral lipids prior to use as delivery agents. Neutral lipids include, but are not limited to, lecithins; [phosphotidylethanolamine] phosphatidylethanolamine; phosphatidylethanolamines, such as DOPE (dioleoylphosphatidylethanolamine), DPPE (dipalmitoylphosphatidylethanolamine), [dipalmiteoylphosphatidylethanolamine,] POPE (palmitoyloleoylphosphatidylethanolamine) and distearoylphosphatidylethanolamine; [phosphotidylcholine] phosphatidylcholine; phosphatidylcholines, such as DOPC ([dioleoylphosphidylcholine] dioleoylphosphatidylcholine), DPPC (dipalmitoylphosphatidylcholine), POPC (palmitoyloleoylphosphatidylcholine) and distearoylphosphatidylcholine; fatty acid esters; glycerol esters; sphingolipids; cardiolipin; cerebrosides; and ceramides; and mixtures thereof. Neutral lipids also include cholesterol and other 3βOH-sterols.

Please amend the paragraph beginning on page 20, line 24, through page 25, line 18, as follows:

Examples of cationic lipid compounds include, but are not limited to: Lipofectin (Life Technologies, Inc., Burlington, Ont.)(1:1 (w/w) formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and dioleoylphosphatidylethanolamine (DOPE)[)]; LipofectAMINE (Life Technologies, Burlington, Ont., see U.S. Patent No. 5,334,761) (3:1 (w/w) formulation of polycationic lipid 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA) and [dioleoyl phosphatidylethanolamine] dioleoylphosphatidylethanolamine (DOPE)[)], LipofectAMINE PLUS (Life Technologies, Burlington, Ont. see U.S. Patent Nos.

5,334,761 and 5,736,392; see, also U.S. Patent No. 6,051,429) (LipofectAmine and Plus reagent), LipofectAMINE 2000 (Life Technologies, Burlington, Ont.; see also International PCT application No. WO 00/27795) (Cationic lipid), Effectene (Qiagen, Inc., Mississauga, Ontario) (Non liposomal lipid formulation), Metafectene (Biontex, Munich, Germany) (Polycationic lipid), Eu-fectins (Promega Biosciences, Inc., San Luis Obispo, CA) (ethanolic cationic lipids numbers 1 through 12: $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$, $C_{88}H_{178}N_8O_4S_2.4CF_3CO_2H$, $C_{40}H_{84}NO_3P.CF_3CO_2H$, $C_{50}H_{103}N_7O_3.4CF_3CO_2H$, $C_{55}H_{116}N_8O_2.6CF_3CO_2H$, $C_{49}H_{102}N_6O_3.4CF_3CO_2H$, $C_{44}H_{89}N_5O_3.2CF_3CO_2H$, $C_{100}H_{206}N_{12}O_4S_2.8CF_3CO_2H$, $C_{162}H_{330}N_{22}O_9.13CF_3CO_2H$, $C_{43}H_{88}N_4O_2.2CF_3CO_2H$, $C_{43}H_{88}N_4O_3.2CF_3CO_2H$, $C_{41}H_{78}NO_8P$); Cytofectene (Bio-Rad, Hercules, CA) (mixture of a cationic lipid and a neutral lipid), GenePORTER (Gene Therapy Systems Inc., San Diego, CA) (formulation of a neutral lipid (Dope) and a cationic lipid) and FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) (Multi-component lipid based non-liposomal reagent).

Please amend the paragraphs beginning on page 44, line 3, through page 46, line 3, as follows:

For preparation of purified genomic DNA, sorted chromosome samples were brought to 0.5% SDS, 50 mm EDTA and 100 μg/ml Proteinase K, then incubated for 18 hours at 50°C. 1 μl of a 20 mg/ml glycogen solution (Boehringer Mannheim) was added to each sample, followed by extraction with an equal volume of Phenol: Chloroform: Isoamyl Alcohol (25:24:1). After centrifugation at 21,000Xg for 10 min, the aqueous phases were transferred to fresh microfuge tubes and were re-extracted as above. 0.2 volumes of 10 M NH_4OAC, 1 μl of 20 mg/ml glycogen and 1 volume of iso-propanol were added to the twice extracted aqueous phases which were then vortexed and centrifuged for 15 minutes at 30,000Xg (at room [temperautre]<u>temperature</u>). Pellets were washed with 200 μl of 70% ethanol and re-centrifuged as above.

The washed pellets were air-dried then resuspended in 5mM Tris-Cl, pH 8.0 at 0.5-2X10^6 chromosome equivalents/μl.

PCR was carried out on DNA prepared from sorted chromosome samples essentially as described (see, Co *et al.* (2000) *Chromosome Research* *8*:183-191) using primers sets specific for EGFP and RAPSYN. Briefly, 50 μl PCR reactions were carried out on genomic DNA equivalent to 10,000 or 1000 chromosomes in a solution containing 10 mM Tris-Cl, pH 8.3, 50mM KCl, 200 μM dNTPs, 500 nM of forward and reverse primers, 1.5 mM $MgCl_2$, 1.25 units Taq polymerase (Ampli-Taq, Perkin-Elmer Cetus, CA). Separate reactions were carried out for each primer set. The reaction conditions were as follows: one cycle of 10 min. at 95°C, then 35 cycles of 1 min. <u>at</u> 94°C, 1 min. <u>at</u> 55°C, 1 min <u>at</u> 72°C, and finally one cycle of 10 min at 72°C. After completion the samples were held at 4°C until analyzed by agarose gel electrophoresis using the following primers (SEQ ID Nos. 1-4, respectively):

EGFP forward primer 5'-cgtccaggagcgcaccatcttctt-3';

EGFP reverse primer 3'-atcgcgcttctcgttggggtcttt-3';

RAPSYN forward primer 5'-aggactgggtggcttccaactcccagacac-3'; and

RAPSYN reverse primer 5'-agcttctcattgctgcgcgccaggttcagg-3'.

All primers were obtained from Canadian Life Technologies, Burlington, ON.

EXAMPLE 2

Preparation of Cationic vesicles

Vesicles were prepared at a lipid concentration of 700 [nmoles]<u>nmol</u>/ml lipid (cationic lipid/DOPE 1:1) as follows. In a glass tube (10ml) 350 [nmoles]<u>nmol</u> cationic lipid (SAINT-2) was mixed with 350[nmoles]<u>nmol</u> [dioleylphosphoethanolamine] <u>dioleoylphosphatidylethanolamine</u> (DOPE), both solubilized in an organic solvent (Chloroform, Methanol or Chloroform/Methanol 1:1, v/v). [Diphosphatidylethanolamine] <u>Dioleoylphosphatidylethanolamine</u> (DOPE; Avanti Polar Lipids, Alabaster, AL) forms inverse hexagonal phases in a membrane and weakens the membrane. Other effectors that may be used are *cis*-unsaturated [phosphoethanolamines] <u>phosphatidylethanolamines</u>, *cis*-

unsaturated fatty acids, and cholesterol. *Cis*-unsaturated phosphatidylcholines are less effective.

The solvent was evaporated under a stream of nitrogen (15 min/ 250 μl solvent at room temperature). The remaining solvent was removed totally by drying the lipid for 15 min in [an] a desiccator under high vacuum from a vacuum pump. To the dried mixture was added l ml ultrapure water. This was vortexed vigorously for about 5 min. The resulting solution was sonicated in an ultrasonication bath (Laboratory Supplies Inc. NY) until a clear solution was obtained. The resulting suspension contained a population of unilamellar vesicles with a size distribution between 50 to 100 nm.

EXAMPLE 3

Preparation of Cationic vesicles via alcoholic injection

In a glass tube (10ml) 350 [nmoles]nmol cationic lipid (Saint-2) was mixed with 350 [nmoles]nmol DOPE, both solubilized in an organic solvent (chloroform, methanol or chloroform/methanol 1/1). The solvent was evaporated under a stream of nitrogen (15 min/ 250 μl solvent at room temperature). The remaining solvent was removed totally by drying the lipid for 15 min under high vacuum. This was then reconstituted in 100 μl pure ethanol.

Please amend the paragraphs beginning on page 50, line 8, through page 51, line 4, as follows:

Transfection of the cells was performed as follows. The medium was removed from the cells, and the cells were washed twice with HBSS (Hanks balanced salt solution without Phenol Red (Gibco BRL, UK)) at 37°C. Then 500μl HBSS at 37°C was added per well, followed by 10 μl of the freshly prepared vesicle solution (prepared in Example 2) to yield a final concentration of 23.3 [nmole]nmol/ml.

Alternatively, the medium was removed from the cells, and the cells were washed twice with HBSS. 500 μl HBSS/lipid solution at 37°C was added to each well. The HBSS/lipid solution was prepared by adding 1 μl ethanolic lipid

solution (prepared as described above) to 500 μl HBSS under vigorous vortexing. The plates were then sealed with parafilm tape and shaken gently at room temperature for 30 min. After incubation, ultrasound was applied at an output energy of 0.5 Watt/cm^2 for 60 sec through the bottom of the plate to the cells. The ultrasound was mediated by an ultrasound gel (Aquasonic 100, Parker, NJ) between transducer and plate. The ultrasound was applied with an ImaRx Sonoporator 100. Immediately after applying ultrasound one GFP chromosome per seeded cell (2 x 10^5 - 5 x 10^5) (prepared in Example 1) was added. The plate was then sealed again and shaken gently for 1 h at room temperature. After the incubation 1ml medium (CHO-S-SFM 2 with 10% Fetal Calf Serum, 10000 μg/ml Penicillin and 10000 μg/ml Streptomycin Gibco BRL, Paisley, UK) was added to each well and the cells were incubated for 24 h at 37°C. The cells were then washed with medium,[and] 1 ml medium was added, and the cells were incubated at 37° for another 24 h. Detection of expressed genes was then assayed by microscopy or detection of the transferred chromosome by FISH analysis.

The negative control was performed in the same way, but with no chromosomes added to the cells.

Please amend the paragraph on page 51, lines 11-17, as follows:

B. Ultrasound mediated transfection of Hep-G2 cells with Saint-2

Hep-G2 cells were grown at 37°C, 5% CO_2, in DMEM with 4500 mg/l Glucose, with Pyridoxine/[HCL]<u>HCl</u>, 10% Fetal Calf Serum, 10000 μg/ml Streptomycin and 1000 μg/ml Penicillin. Between 2 x 10^5 and 5 x 10^5 cells were plated onto sterile glass slides in a 12 wells plate 24 hours before usage.

Please amend the paragraph on page 51, lines 24-29, as follows:

C. Ultrasound mediated transfection of A9 cells with Saint-2

A9 cells were grown at 37°C, 5% CO_2, in DMEM with 4500 mg/l Glucose, with Pyridoxine/[HCL]<u>HCl</u>, 10% Fetal Calf Serum, 10000 μg/ml

Streptomycin and 10000 μg/ml Penicillin (GIBCO BRL, Paisley, UK). Between 2 x 10^5 and 5 x 10^5 cells were plated onto sterile glass slides in a 12 well plate 24 h before usage.

Please amend the paragraphs beginning on page 52, line 7, through page 54, line 6, as follows:

EXAMPLE 7

A flow cytometry technique for measuring delivery of artificial chromosomes

Production cells lines (see Example 1) were grown in MEM medium (Gibco BRL) with 10% fetal calf serum (Can Sera, Rexdale ON) with 0.168 μg/ml hygromycin B (Calbiochem, San Diego, CA). [Iododeoxyurine] Iododeoxyuridine or [Bromodeoxyurdine] Bromodeoxyuridine [were] was added directly to culture medium of the production cell line (CHO E42019) in the exponential phase of growth. Stock [Iododeoxyurdine] Iododeoxyuridine was made in tris base pH 10[.], [Bromodeoxyurdine] Bromodeoxyuridine stocks in PBS. Final concentrations of 0.05-1 μM for continuous label of 20-24 hours of 5-50 μM with 15 minute pulse. After 24 hours, exponentially growing cells were blocked in mitosis with colchicine (1.0 μg/ml for 7 hours before harvest. Chromosomes were then isolated and stained with Hoechst 33258 (2.5 μg/ml) and chromomycin A3 (50 μg/ml). Purification of artificial chromosomes was performed using a FACS Vantage flow cytometer (Becton Dickinson Immunocytometry systems, San Jose, CA). Chromomycin A3 was excited with the primary laser set at 457 nm, with emission detected using 475 nm long pass filter. Hoechst was excited by the secondary UV laser and emission detected using a 420/44 nm band-pass filter. Both lasers had an output of 150 mW. Bivariate distribution showing cell karyotype was accumulated from each sort. ACes were gated from other chromosomes and sorted. Condensing agents (hexylene glycol, spermine, and spermidine) were added to the sheath buffer to maintain condensed intact chromosome after sorting. IdU labeling index of sorted chromosomes was determined microscopically. Aliquot (2-10 μl) of sorted

chromosomes was fixed in 0.2% formaldehyde solution for 5 minutes before being dried on clean microscopic slide. Microscope sample was fixed with 70% ethanol. Air-dried slide was denatured in coplin jar with [2NHCL]2N HCl for 30 minutes at room temperature and washed 2-3 times with PBS. Non specific binding was blocked with PBS and 4% BSA or serum for minimum of 10 minutes. A 1/5 dilution of FITC conjugated IdU/BrdU antibody (Becton Dickinson) with a final volume of 60-100 μl was applied to slide. Plastic strips, Durra seal (Diversified Biotech, Boston, MA) were overlaid on slides, and slides were kept in dark at 4%C in humidified covered box for 8-24 hours. DAPI (Sigma) 1 μg/ml in Vectorshield was used as counterstain. Fluorescence was detected using Zeiss axioplan 2 microscope equipped for epiflorescence. Minimum of 100 chromosomes was scored for determining % labeled. Unlabeled chromosomes were used as negative control.

The day before the transfection, trypsinize V79-4 (Chinese Hamster Lung fibroblast) cells and plate at 250,000 into a 6 well petri dish in 4 [mls]ml DMEM (Dulbecco's Modified Eagle Medium, Life Technologies) and 10% FBS (Can Sera Rexdale ON). The protocol was modified for use with LM (tk-) cell line by plating 500,000 cells. Lipid or dendrimer reagent was added to 1 X10^6 ACes sorted in ~800 μl sort buffer. Exemplary protocol variations are set forth in Table 1. Chromosome and transfection agents were mixed gently. Complexes were added to cells drop-wise and plate swirled to mix. Plates were kept at 37°C in a 5% CO_2 incubator for specified transfection time. The volume in a well was then made up to 4-5 ml with DMEM and 10% FBS. Recipient cells left for 24 hours at 37°C in a 5% CO_2 incubator. Trypsinize transfected cells. Samples to be analyzed for IdU labeled chromosome delivery are fixed in cold 70% ethanol and stored at -20°C, [tp]to be ready for IdU antibody staining. Samples to be grown for colony selection are counted and then transferred to 10-cm dishes at densities of 10,000 and 100,000 cells in duplicate with remaining cells put in a 15 cm dish. After 24 hours, selection medium containing of DMEM and 10% FBS with 0.7 mg/ml hygromycin B, # 400051

(Calbiochem San Diego, CA) is added. Selection medium is changed every 2-3 days. This concentration of hygromycin B kills the wild type cells after selection for 7 days. At 10-14 days colonies are expanded and then screened by FISH for intact chromosome transfer and assayed for beta galactosidase expression.

Please amend the paragraphs beginning on page 55, line 7, through page 56, line 8, as follows:

IDU ANTIBODY LABELING

A standard BrdU staining flow cytometry protocol (Gratzer et al. Cytometry (1981);6:385-393) is used except with some modifications at neutralization step, the presence of detergent during denaturation and the composition of blocking buffer. Between each step samples are centrifuged at 300 g for 7-10 minutes and supernatant removed. Samples of 1-2 million cells are fixed in 70% cold ethanol. Cells are then denatured in 1-2 ml of 2N [HCL]HCl plus 0.5% triton X for 30 minutes at room temperature. Sample undergoes 3-4 washes with cold DMEM until indictor is neutral. Final wash with cold DMEM plus 5% FBS. Blocking/permeabilization buffer containing PBS, 0.1% triton X and 4% FBS is added for 10-15 minutes before pelleting sample by centrifugation. Add 20 μl of IdU/BrdU FITC conjugated B44 clone antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA) to pellet and leave for 2 hours at room temperature in the dark with agitation every 30 minutes. Wash cells with block/permeabilization buffer and resuspend in PBS for flow analysis.

FLOW CYTOMETRY DETECTION OF FLUORESCENT [IDUrdD]IDUrd LABELED ACes

Percentage of transfected cells containing IdU labeled ACes was determined using a flow cytometry with an argon laser turned to 488 nm at 400 mW. FITC fluorescence was collected through a standard FITC 530/30-nm band pass filter. Cell populations were gated on the basis of side scatter versus forward scatter to exclude debris and doublets. Data [was]were accumulated

(15,000 events) to form bivariate channel distribution showing forward scatter versus green fluorescence (IdU-FITC). The fluorescence level at which cells were determined to be positive was established by visual inspection of the histogram of negative control cells, such that approximately 1% appeared in the positive region.

Results:

The transfection delivery results of IdU labeled ACes are set forth in Table 2.

IN THE CLAIMS:

Please amend the claims 6, 8, 14, 36, 39, 42, 51, 53, 59, 67, 71, 79, 87, 89, 94, 96, 102, 104, 110, 112, 118, 120, 127, 133, 138, 140, 141, and 143 as follows:

6. (Amended) The method of claim 1, wherein the nucleic acid molecule is a natural chromosome, an artificial chromosome, a fragment of a chromosome that is greater than about 0.6 megabase or naked DNA that is greater than about 0.6 [megabases]<u>megabase</u>.

8. (Amended) The method of [claims]<u>claim</u> 1, wherein the nucleic acid molecule is an artificial chromosome expression [systems]<u>system</u> ([Aces]<u>ACes</u>).

14. (Amended) The method of claim 12, wherein the delivery agent is a composition that comprises one or more cationic compounds, wherein the compound is selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), dioleoylphosphatidylethanolamine (DOPE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), [dioleoyl phosphatidylethanolamine (DOPE),] $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$, $C_{88}H_{178}N_8O_4S_2.4CF_3CO_2H$, $C_{40}H_{84}NO_3P.CF_3CO_2H$, $C_{50}H_{103}N_7O_3.4CF_3CO_2H$, $C_{55}H_{116}N_8O_2.6CF_3CO_2H$, $C_{49}H_{102}N_6O_3.4CF_3CO_2H$, $C_{44}H_{89}N_5O_3.2CF_3CO_2H$, $C_{100}H_{206}N_{12}O_4S_2.8CF_3CO_2H$, $C_{41}H_{78}NO_8P)$ $C_{162}H_{330}N_{22}O_9.13CF_3CO_2H$, $C_{43}H_{88}N_4O_2.2CF_3CO_2H$,

$C_{43}H_{88}N_4O_3.2CF_3CO_2H$, and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl) pyridinium chloride.

36. (Amended) The method of claim 34, wherein the delivery agent is a composition that comprises one or more cationic compounds, wherein the compound is selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), dioleoylphosphatidylethanolamine (DOPE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), [dioleoyl phosphatidylethanolamine (DOPE),] $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$, $C_{88}H_{178}N_8O_4S_2.4CF_3CO_2H$, $C_{40}H_{84}NO_3P.CF_3CO_2H$, $C_{50}H_{103}N_7O_3.4CF_3CO_2H$, $C_{55}H_{116}N_8O_2.6CF_3CO_2H$, $C_{49}H_{102}N_6O_3.4CF_3CO_2H$, $C_{44}H_{89}N_5O_3.2CF_3CO_2H$, $C_{100}H_{206}N_{12}O_4S_2.8CF_3CO_2H$, $C_{41}H_{78}NO_8P$), $C_{162}H_{330}N_{22}O_9.13CF_3CO_2H$, $C_{43}H_{88}N_4O_2.2CF_3CO_2H$, $C_{43}H_{88}N_4O_3.2CF_3CO_2H$, and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl) pyridinium chloride.

39. (Amended) The method of claim 34, wherein the nucleic acid molecule is selected from the group consisting of an artificial chromosome, a artificial chromosome expression system ([Aces]<u>ACes</u>) and a natural chromosome or a fragment thereof that is greater than at least about 0.6 megabase.

42. (Amended) The method of claim 41, wherein the ultrasound energy is applied to the cell at between about 0.1 and 1 [watts/cm^2,]<u>watt/cm^2,</u> for about 30 seconds to about 5 minutes.

51. (Amended) The method of claim 48, wherein the delivery agent is a composition that comprises one or more cationic compounds, wherein the compound is selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), dioleoylphosphatidylethanolamine (DOPE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), [dioleoyl phosphatidylethanolamine (DOPE),] $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$, $C_{88}H_{178}N_8O_4S_2.4CF_3CO_2H$, $C_{40}H_{84}NO_3P.CF_3CO_2H$, $C_{50}H_{103}N_7O_3.4CF_3CO_2H$, $C_{55}H_{116}N_8O_2.6CF_3CO_2H$,

$C_{49}H_{102}N_6O_3.4CF_3CO_2H$, $C_{44}H_{89}N_5O_3.2CF_3CO_2H$, $C_{100}H_{206}N_{12}O_4S_2.8CF_3CO_2H$, $C_{41}H_{78}NO_8P$), $C_{162}H_{330}N_{22}O_9.13CF_3CO_2H$, $C_{43}H_{88}N_4O_2.2CF_3CO_2H$, $C_{43}H_{88}N_4O_3.2CF_3CO_2H$, and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl) pyridinium chloride.

53. (Amended) The method of claim 48, wherein the nucleic acid molecule is greater than about 1 [megabases]<u>megabase</u>.

59. (Amended) The method of claim 58, wherein the cationic lipid composition comprises 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA) and [dioleoyl phosphatidyl-ethanolamine] <u>dioleoylphosphatidylethanolamine</u> (DOPE).

67. (Amended) The method of claim 66, wherein the cationic compound and the nucleic acid molecule mixture [is] <u>are</u> applied locally.

71. (Amended) The method of claim 65, wherein the nucleic acid molecule is selected from the group consisting of an artificial chromosome, [a] <u>an</u> artificial chromosome expression system ([Aces]<u>ACes</u>), a natural chromosome or a fragment thereof that is greater than at least about 0.6 megabase.

79. (Amended) The method of claim 73, wherein the delivery agent is a composition that comprises one or more cationic compounds, wherein the compound is selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), dioleoylphosphatidylethanolamine (DOPE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), [dioleoyl phosphatidylethanolamine (DOPE),] $C_{52}H_{106}N_6O_4{\cdot}4CF_3CO_2H$, $C_{88}H_{178}N_8O_4S_2.4CF_3CO_2H$, $C_{40}H_{84}NO_3P.CF_3CO_2H$, $C_{50}H_{103}N_7O_3.4CF_3CO_2H$, $C_{55}H_{116}N_8O_2.6CF_3CO_2H$, $C_{49}H_{102}N_6O_3.4CF_3CO_2H$, $C_{44}H_{89}N_5O_3.2CF_3CO_2H$, $C_{100}H_{206}N_{12}O_4S_2.8CF_3CO_2H$, $C_{41}H_{78}NO_8P$), $C_{162}H_{330}N_{22}O_9.13CF_3CO_2H$, $C_{43}H_{88}N_4O_2.2CF_3CO_2H$, $C_{43}H_{88}N_4O_3.2CF_3CO_2H$, and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl) pyridinium chloride.

87. (Amended) The method of claim 85, wherein the compound is selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), dioleoylphosphatidylethanolamine (DOPE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), [dioleoyl phosphatidylethanolamine (DOPE),] $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$, $C_{88}H_{178}N_8O_4S_2.4CF_3CO_2H$, $C_{40}H_{84}NO_3P.CF_3CO_2H$, $C_{50}H_{103}N_7O_3.4CF_3CO_2H$, $C_{55}H_{116}N_8O_2.6CF_3CO_2H$, $C_{49}H_{102}N_6O_3.4CF_3CO_2H$, $C_{44}H_{89}N_5O_3.2CF_3CO_2H$, $C_{41}H_{78}NO_8P$), $C_{100}H_{206}N_{12}O_4S_2.8CF_3CO_2H$, $C_{162}H_{330}N_{22}O_9.13CF_3CO_2H$, $C_{43}H_{88}N_4O_2.2CF_3CO_2H$, $C_{43}H_{88}N_4O_3.2CF_3CO_2H$ and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl) pyridinium chloride.

89. (Amended) The method of claim 84, wherein the [plant] <u>cell</u> is a plant cell or an animal cell.

94. (Amended) The method of claim 92, wherein the compound is selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), dioleoylphosphatidylethanolamine (DOPE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), [dioleoyl phosphatidylethanolamine (DOPE),] $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$, $C_{88}H_{178}N_8O_4S_2.4CF_3CO_2H$, $C_{40}H_{84}NO_3P.CF_3CO_2H$, $C_{50}H_{103}N_7O_3.4CF_3CO_2H$, $C_{55}H_{116}N_8O_2.6CF_3CO_2H$, $C_{49}H_{102}N_6O_3.4CF_3CO_2H$, $C_{44}H_{89}N_5O_3.2CF_3CO_2H$, $C_{41}H_{78}NO_8P$), $C_{100}H_{206}N_{12}O_4S_2.8CF_3CO_2H$, $C_{162}H_{330}N_{22}O_9.13CF_3CO_2H$, $C_{43}H_{88}N_4O_2.2CF_3CO_2H$, $C_{43}H_{88}N_4O_3.2CF_3CO_2H$ and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl) pyridinium chloride.

96. (Amended) The method of claim 92, wherein the [plant] <u>cell</u> is a plant cell or an animal cell.

102. (Amended) The method of claim 100, wherein the compound is selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), dioleoylphosphatidylethanolamine (DOPE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-

propanaminiumtrifluoroacetate (DOSPA), [dioleoyl phosphatidylethanolamine (DOPE),] $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$, $C_{88}H_{178}N_8O_4S_2.4CF_3CO_2H$, $C_{40}H_{84}NO_3P.CF_3CO_2H$, $C_{50}H_{103}N_7O_3.4CF_3CO_2H$, $C_{55}H_{116}N_8O_2.6CF_3CO_2H$, $C_{49}H_{102}N_6O_3.4CF_3CO_2H$, $C_{44}H_{89}N_5O_3.2CF_3CO_2H$, $C_{41}H_{78}NO_8P$), $C_{100}H_{206}N_{12}O_4S_2.8CF_3CO_2H$, $C_{162}H_{330}N_{22}O_9.13CF_3CO_2H$, $C_{43}H_{88}N_4O_2.2CF_3CO_2H$, $C_{43}H_{88}N_4O_3.2CF_3CO_2H$ and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl) pyridinium chloride.

104. (Amended) The method of claim 100, wherein the [plant] <u>cell</u> is a plant cell or an animal cell.

110. (Amended) The method of claim 108, wherein the compound is selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), dioleoylphosphatidylethanolamine (DOPE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), [dioleoyl phosphatidylethanolamine (DOPE),] $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$, $C_{88}H_{178}N_8O_4S_2.4CF_3CO_2H$, $C_{40}H_{84}NO_3P.CF_3CO_2H$, $C_{50}H_{103}N_7O_3.4CF_3CO_2H$, $C_{55}H_{116}N_8O_2.6CF_3CO_2H$, $C_{49}H_{102}N_6O_3.4CF_3CO_2H$, $C_{44}H_{89}N_5O_3.2CF_3CO_2H$, $C_{41}H_{78}NO_8P$), $C_{100}H_{206}N_{12}O_4S_2.8CF_3CO_2H$, $C_{162}H_{330}N_{22}O_9.13CF_3CO_2H$, $C_{43}H_{88}N_4O_2.2CF_3CO_2H$, $C_{43}H_{88}N_4O_3.2CF_3CO_2H$ and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl) pyridinium chloride.

112. (Amended) The method of claim 108, wherein the [plant] <u>cell</u> is a plant cell or an animal cell.

118. (Amended) The method of claim 116, wherein the compound is selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), dioleoylphosphatidylethanolamine (DOPE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), [dioleoyl phosphatidylethanolamine (DOPE),] $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$, $C_{88}H_{178}N_8O_4S_2.4CF_3CO_2H$, $C_{40}H_{84}NO_3P.CF_3CO_2H$, $C_{50}H_{103}N_7O_3.4CF_3CO_2H$, $C_{55}H_{116}N_8O_2.6CF_3CO_2H$, $C_{49}H_{102}N_6O_3.4CF_3CO_2H$, $C_{44}H_{89}N_5O_3.2CF_3CO_2H$, $C_{41}H_{78}NO_8P$),

$C_{100}H_{206}N_{12}O_4S_2.8CF_3CO_2H$, $C_{162}H_{330}N_{22}O_9.13CF_3CO_2H$, $C_{43}H_{88}N_4O_2.2CF_3CO_2H$, $C_{43}H_{88}N_4O_3.2CF_3CO_2H$ and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl) pyridinium chloride.

120. (Amended) The method of claim 116, wherein the [plant] <u>cell</u> is a plant cell or an animal cell.

127. (Amended) The method of claim 124, wherein the [plant] <u>cell</u> is a plant cell or an animal cell.

133. (Amended) The method of claim 131, wherein the [plant] <u>cell</u> is a plant cell or an animal cell.

138. (Amended) The method of claim 131, wherein the compound is selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), dioleoylphosphatidylethanolamine (DOPE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), [dioleoyl phosphatidylethanolamine (DOPE),] $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$, $C_{88}H_{178}N_8O_4S_2.4CF_3CO_2H$, $C_{40}H_{84}NO_3P.CF_3CO_2H$, $C_{50}H_{103}N_7O_3.4CF_3CO_2H$, $C_{55}H_{116}N_8O_2.6CF_3CO_2H$, $C_{49}H_{102}N_6O_3.4CF_3CO_2H$, $C_{44}H_{89}N_5O_3.2CF_3CO_2H$, $C_{41}H_{78}NO_8P$), $C_{100}H_{206}N_{12}O_4S_2.8CF_3CO_2H$, $C_{162}H_{330}N_{22}O_9.13CF_3CO_2H$, $C_{43}H_{88}N_4O_2.2CF_3CO_2H$, $C_{43}H_{88}N_4O_3.2CF_3CO_2H$ and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl) pyridinium chloride.

140. (Amended) A kit for delivering nucleic acids into cells, comprising:

a delivery agent that comprises a composition comprising a delivery agent;

reagents for performing [sonorporation] <u>sonoporation</u> or electroporation; and

optionally instructions for delivering nucleic acids into cells.

141. (Amended) The kit of claim 140, further comprising a [compositions] <u>composition</u> comprising an artificial chromosome.

143. (Amended) The kit of claim 142, wherein the compound is selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl-

ammonium chloride (DOTMA), dioleoylphosphatidylethanolamine (DOPE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), [dioleoyl phosphatidylethanolamine (DOPE),] $C_{52}H_{106}N_6O_4 \cdot 4CF_3CO_2H$, $C_{88}H_{178}N_8O_4S_2.4CF_3CO_2H$, $C_{40}H_{84}NO_3P.CF_3CO_2H$, $C_{50}H_{103}N_7O_3.4CF_3CO_2H$, $C_{55}H_{116}N_8O_2.6CF_3CO_2H$, $C_{49}H_{102}N_6O_3.4CF_3CO_2H$, $C_{44}H_{89}N_5O_3.2CF_3CO_2H$, $C_{41}H_{78}NO_8P$), $C_{100}H_{206}N_{12}O_4S_2.8CF_3CO_2H$, $C_{162}H_{330}N_{22}O_9.13CF_3CO_2H$, $C_{43}H_{88}N_4O_2.2CF_3CO_2H$, $C_{43}H_{88}N_4O_3.2CF_3CO_2H$ and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl) pyridinium chloride.